

1 **No transfer of the non-regulated mycotoxins, beauvericin and enniatins, from**
2 **feeds to farmed fish reared on plant-based diets**

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22 **Abstract**

23 Concern about the risk of exposure to emerging plant-derived mycotoxins such as beauvericin and
24 enniatins has been addressed by the European Commission who requested the European Food
25 Safety Authority for a scientific opinion on their risk to human and animal health. The studied
26 mycotoxins were found in feeds with enniatin B and beauvericin at average concentrations of 19.9
27 µg/kg and 30 µg/kg, respectively. In all cases, concentrations of all the mycotoxins analyzed were
28 below quantification limits (< 0.1 µg/kg) in fish samples (n = 82). The present work provides
29 comprehensive and traceable data of emerging mycotoxins in plant-based aquafeeds and fish reared
30 on the feeds, responding to increasing concerns about safety of farmed fish fed on sustainable feeds.
31 On the basis of data reported, there was no transfer of the emerging mycotoxins, beauvericin and
32 enniatins, from feeds to fish and so, no risk for human consumption.

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34 **Keywords:** mycotoxins, fish, feed, liquid chromatography, mass spectrometry, transfer, Atlantic
35 salmon, sea bream.

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45 **1. Introduction**

46 Fish consumption has shown continuous growth due to increasing demand, world market
47 availability, and consumer trends related to concerns for healthy eating (FAO, 2014). This
48 expansion, combined with the limited availability of marine raw materials, fishmeal and fish oil, has
49 forced the European aquafeed industry to explore alternative, sustainable sources of protein and
50 lipid for feeds. Consequently, the most important European farmed fish species, including Atlantic
51 salmon (*Salmo salar*) and gilthead sea bream (*Sparus aurata*), are currently being farmed
52 successfully using feeds with a very high inclusion of plant-based ingredients, plant meals and
53 vegetable oils, with only a low inclusion level of marine ingredients (Liland et al., 2013; Shepherd
54 et al., 2017; Simó-Mirabet et al., 2018). However, these achievements have also brought concerns
55 about the safety of market fish, of relevance not only for European food authorities and farmers, but
56 also consumers. These concerns about potential risks to human health and animal welfare led to
57 considerable research to determine what contaminants are of relevance in aquaculture based on
58 changing feed formulations over the last decade or more (Nácher-Mestre et al., 2018; Simó-Mirabet
59 et al., 2018).

60 The use of plant-based feeds can introduce contaminants that were not previously associated with
61 fish farming utilizing traditional marine feed ingredients. Mycotoxins represent an important group
62 of naturally-derived plant contaminants found world-wide in cereals, and consequently introduced
63 into the new, alternative and sustainable aquafeeds. Concern about the risk of exposure to these
64 mycotoxins has been partially addressed (EC, 2002; EFSA, 2011; EU, 2006) in research conducted
65 primarily to protect consumers, but also animal health, in the context of improvement of food safety
66 and farming industry efficiency. Anater et al. collected the presence of residues of mycotoxins in
67 fish and estimated risks for public health (Anater et al., 2016). These analysis in wild European sea
68 bass (*Dicentrarchus labrax L.*), sea bream (*Sparus aurata*), rainbow trout (*Oncorhynchus mykiss*)
69 and tilapia (*Oreochromis niloticus*), under natural occurrence, resulted in non-detectable levels of

70 mycotoxins in muscle (Anater et al., 2016). Moreover, recent studies carried out on farmed Atlantic
71 salmon and gilthead sea bream revealed that current regulated mycotoxins were not transferred
72 from feeds to the edible fillets of the fish after representative feeding trials (Nácher-Mestre et al.,
73 2015; Johny et al., 2019). However, other studies reported the presence of the mycotoxins, enniatins
74 A1, B and B1, in market sea bass and sea bream fillets and also in liver, head and viscera (Tolosa et
75 al., 2013, 2014). The above cited studies could be considered as the current state-of-the-art of
76 knowledge regarding mycotoxins in farmed fish, but this requires to be extended to other European
77 farmed fish species, including an assessment of the potential transfer of mycotoxins from feed to the
78 edible part of the fish in controlled feeding trials. This accounts in particular to new and emerging
79 mycotoxins that have not yet been assessed.

80 In fact, a European Food Safety Authority (EFSA) Scientific Opinion has already indicated the
81 potential risks to human and terrestrial animal health due to the presence of emerging mycotoxins,
82 beauvericin and enniatins, in food and feed (EFSA, 2014). However, exposure estimated to
83 beauvericin and enniatins for farmed fish or their potential feed-to-fillet transfer have not been
84 made in the EFSA opinion (EFSA, 2014). These mycotoxins are more lipophilic than currently
85 regulated mycotoxins and so they could be potentially deposited and accumulated in edible fillets
86 and other essential fish organs.

87 Taking into account the above justification, the development of analytical strategies to monitor and
88 quantify these natural toxins in feeds and fish fed plant-based diets should be carried out and,
89 indeed, is expected by Authorities (EFSA, 2014). In addition, data on emerging mycotoxins in
90 sustainable feeds and fish fed these feeds is required by the European aquaculture industry and
91 regulatory authorities to increase relevant knowledge for future actions and regulations. For this
92 purpose, the use of liquid chromatography coupled to mass spectrometry working in tandem mode
93 (LC-MS/MS) are consistent with EFSA recommendations as matrix effects can be better addressed
94 with this instrumentation, and these methods are able to perform reliable quantification at
95 concentrations below 1 µg/kg (EFSA, 2014). Thus, LC-MS/MS makes feasible the transfer of

96 acquired knowledge of relevance to emerging mycotoxins in farmed fish fed plant-based diets to the
97 European aquaculture industry. The present study utilized ultra-high performance liquid
98 chromatography-tandem mass spectrometry (UHPLC-MS/MS) to quantify levels of the emerging
99 mycotoxins, enniatins and beauvericin, in feeds formulated with high levels of plant-derived
100 ingredients and in Atlantic salmon and gilthead sea bream reared to market size on these feeds.

101

102 **2. Materials and methods**

103 *2.1. Reagents and chemicals*

104 HPLC-grade methanol (MeOH), HPLC-supergradient acetonitrile, formic acid (>98%),
105 ammonium acetate (NH₄Ac), reagent grade were obtained from Flucka (Buchs, Switzerland).
106 HPLC-grade water was obtained from a Milli-Q water purification system (Millipore Ltd., Bedford,
107 MA, USA). Analytical standards for the emerging mycotoxins ($\geq 95\%$, HPLC) beauvericin (BEA),
108 enniatin A (EnA), enniatin A1 (EnA1), enniatin B (EnB) and enniatin B1 (EnB1) were purchased
109 from Sigma Aldrich (Gillingham, UK).

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111 *2.2. Samples*

112 A total of 21 non-spiked feeds (17 for Atlantic salmon and 4 for sea bream) formulated with
113 high percentages of plant proteins and oils were analyzed from controlled feeding trials conducted
114 at the University of Stirling (UoS, Scotland), Institute of Marine Research (IMR, Norway) and the
115 Institute of Aquaculture Torre de la Sal (IATS, Spain) within the framework of the EU ARRAINA
116 Project and UK TSB Beans4Feeds project. Briefly, in experimental trials conducted by UoS,
117 Atlantic salmon were fed with 6 different diet formulations (corresponding to 14 feeds), with fish
118 sampled at 3, 6 and 11 months' post application of experimental feeds. A total of 50 samples (each
119 sample was pooled from 5 fillets) were analyzed from dietary treatments, from intermediate and
120 final sampling points, covering juveniles (250 g) to market fish size (2.6 kg). In parallel in this trial,
121 18 samples of whole salmon (pools of two fish) were also analyzed. In addition, two trials with

122 freshwater salmon (parr) and seawater-adapted salmon (post-smolt) were performed by IMR with 3
123 different dietary formulations (3 feeds analyzed), in triplicate tanks/cages over a 11 month period,
124 covering freshwater start feeding salmon (18 g) to seawater transferred post smolt (480 g). At the
125 end of the trial, 10 fish from each cage were sampled and muscle samples were pooled. From those
126 stored, a total of 10 available samples were analyzed. In the case of sea bream trial, juveniles of
127 Atlantic origin (Ferme Marine de Douhet, France) were acclimated for four weeks to the indoor
128 experimental facilities of the Institute of Aquaculture Torre de la Sal (IATS, Spain). During this
129 initial period, fish were fed with a standard diet (Efico YM 568 1.9 mm, BioMar). Then, fish of 13–
130 16 g initial mean body weight were distributed in 2500 L tanks in triplicate groups of 180 fish each.
131 Oxygen content of outlet water remained higher than 75% saturation, and day-length and water
132 temperature followed the natural changes at IATS latitude (40° 5'N; 0° 10'E). Sea bream was fed
133 with 4 different diet formulations (4 feeds analyzed) offered to visual satiety 1–2 times per day and
134 3–6 days per week from May 2013 to October 2014 (18-month feeding trial), according to the
135 changes in fish size and season. At the end of trial, 20 fish of market size (0.8 – 1 kg) were
136 randomly sampled and pools of 5 fish per each dietary condition were analyzed (4 fish samples in
137 total). More specific details of the salmon (De Santis et al., 2016; Hemre et al., 2016; Taylor et al.,
138 2019; Vera et al., 2019) and sea bream (Simó-Mirabet et al., 2018) trials can be found in the
139 previously published studies.

140

141 2.3. *Processing of samples and mycotoxin extraction*

142 The samples of feeds and fish analyzed were collected at appropriate times during and at the
143 end of the feeding trials carried out between 2014 and 2016 (De Santis et al., 2016; Hemre et al.,
144 2016; Simó-Mirabet et al., 2018; Taylor et al., 2019; Vera et al., 2019). Samples of feeds were
145 ground in a blender (Waring Laboratory Science, Winsted, CT, USA) and stored at -40 °C prior to
146 analysis. Whole salmon samples (pools of 2 fish) and salmon and sea bream fillet samples (pools of
147 5 fish) were homogenized in a blender (Waring Laboratory Science) to produce homogenous pates

148 that were stored at -70 °C prior to analysis. Samples of 2.5 g of homogenized feed, fish or fillet
149 were accurately weighed and subsequently extracted with 10 ml acetonitrile using an automatic
150 mechanical shaker at room temperature for 90 min. The extract was then centrifuged (5 min, 4500
151 rpm) followed by 1:4 dilution with water (1:10 dilution in feed matrix) prior analysis by LC-
152 MS/MS.

153

154 2.4. UHPLC-MS/MS

155 A Waters Acquity UPLC system (Waters, Milford, MA, USA) was coupled to a triple
156 quadrupole mass spectrometer (Xevo TQS, Waters, Manchester, UK), using a Z-spray-ESI interface
157 operating in positive ionization mode. The UHPLC separation was performed using an Acquity
158 UPLC® BEH C18 analytical column 50 x 2.1 mm, 1.7 µm particle size (Waters) at a flow rate of
159 400 µL/min. The mobile phase consisted of water and methanol containing both solvents 5 mM
160 ammonium acetate and 0.1 % formic acid as modifiers. Chromatographic separation was carried out
161 in gradient mode, changing the percentage of organic solvent as follows: 0 min, 60 %; 4 min, 90 %;
162 4.1 min, 60 %; 6.0 min, 60 %. The column temperature was set at 40 °C and sample injection
163 volume was 10 µL.

164 Cone voltage and collision energy were optimized by acquisition of full-scan MS and MS/MS
165 spectra of 1 ng/mL targeted mycotoxin reference standard. Three Selected Reaction Monitoring
166 (SRM) transitions using as a precursor ion the $[M+H]^+$ were proposed for quantification (Q_i) and
167 confirmation purposes (q_i). The UHPLC-MS/MS conditions are detailed in Table 1.

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169 2.5. Analytical method validation

170 Method validation was carried out in 3 different matrices (feed, fish fillet and whole fish).
171 Investigated compounds were beauvericin and enniatins A, A1, B and B1 at 1 µg/kg (25 ng/L in
172 extract) and 10 µg/kg (250 ng/L in extract) in feed. For fish fillet and whole fish the methodology
173 was validated at 0.1 µg/kg (6.25 ng/L in extract) and 1 µg/kg (62.5 ng/L in extract).

Linearity of the method was evaluated taking six matrix-matched standard solutions (from 2.5 ng/L to 10000 ng/L) for the 5 selected mycotoxins injected in triplicate. Correlation coefficients higher than 0.99 with residuals lower than 20 % were required for satisfactory linearity. Recoveries and precision (repeatability, expressed as relative standard deviation (RSD) in %), were determined within-day by analyzing spiked blank samples (n = 5) at the spiking levels. Acceptable recoveries and RSDs were established according to SANTE/11945/2015 guidelines for analytical quality control and method validation procedures (expected and desirable recoveries between 70 and 120 % with RSD lower than 20 %) (SANTE, 2015).

Limits of quantification (LOQ) were established at the lowest concentration validated, at 1 µg/kg for feed and 0.1 µg/kg in the case of fillets and whole fish. The specificity of the method was evaluated by analyzing a blank procedure, a processed blank sample and the same blank spiked at the lowest concentration validated for each studied matrix. Satisfactory results were considered when the signal obtained from blank samples was lower than 30 % of the signal at LOQ. Quantification was carried out by means of matrix matched calibration to ensure an accurate determination compensating for matrix effect. Matrix effects were evaluated by comparison of calibration curves slopes when slopes were prepared in neat solvent and in matrix.

$$Matrix\ effect\ (\%) = \left(\frac{Matrix\ calibration\ slope}{Solvent\ calibration\ slope} - 1 \right) \times 100$$

Matrix effects were calculated in percentage. Positive values indicated signal enhancement, whereas negative values were associated to signal suppression. Matrix matched calibration consisted of blank extracts (900 µL) spiked with 100 µL of the corresponding calibration mix standard. Data obtained from analyzed samples was processed both automatically and manually by the TargetLynx application manager (within MassLynx v 4.1; Waters Corporation).

The q_i/Q ratios considered as the ratio between the intensity of the identification transition (q_i) and the quantification transition (Q), were used to additionally confirm peak identity in all samples

199 analyzed (Table 1). According to SANTE/11945/2015 guidelines, q_i/Q transitions were accepted for
200 identification purposes when the q_i/Q ratios were below 30%, in comparison with the experimental
201 q_i/Q value calculated from solvent standards (SANTE, 2015).

202

203 **3. Results and Discussion**

204 *3.1. UHPLC-MS/MS*

205 The use of UHPLC-MS/MS has experimented an increasing use in the last years thanks to
206 their extraordinary sensitivity and the short run time analysis achieved for a limited number of
207 target mycotoxins (Beltrán et al., 2009, 2013). Despite of this fact, limitations for multi-mycotoxins
208 have been found to get enough points per peak in MS/MS data acquisitions (Malachova et al.,
209 2014). In our case, UHPLC with the use of a C18-50mm column working with a flow rate of 400
210 $\mu\text{L}/\text{min}$ allowed the analysis of the five selected mycotoxins in only 6 min run time. Acceptable
211 peak shapes with at least 12 points per peak were obtained for the five mycotoxins studied. This
212 was also feasible with the gradient mode selected, starting with 60% of organic solvent.

213 The addition of modifiers in the mobile phase is crucial to improve the ionization of selected
214 compounds in source and to obtain the maximum sensitivity in target analysis. In the present study,
215 the addition of formic acid, acetic acid and ammonium acetate was studied to test the MS/MS
216 performance and to enhance SRM transitions selecting $[M+H]^+$ and/or $[M+NH_4]^+$ as precursor ions,
217 instead of sodium adducts. These additives are commonly included, in mobile phase and/or
218 extraction solvent, in multi-mycotoxin methods (Beltrán et al., 2009, 2013; de Souza et al., 2013;
219 Malachova et al., 2014; Sulyok et al., 2007). It has also been demonstrated that the use of
220 ammonium acetate in the mobile phase is crucial to promote $[M+H]^+$ instead of sodium adducts in
221 the case of aflatoxins (Beltrán et al., 2009, 2013). Acidic conditions are common in multi-
222 mycotoxin methods and are necessary for the extraction and determination of fumonisins by LC-
223 ESI-MS/MS (Beltrán et al., 2009, 2013; de Souza et al., 2013; Malachova et al., 2014; Sulyok et al.,
224 2007). In the analysis of beauvericin and enniatins, some authors have also applied these modifiers

225 suggesting their use to improve peak shape and analyte response (Decleer et al., 2016; Renaud et
226 al., 2017; Tolosa et al., 2013, 2014, 2019).

227 In the present work, study of the ionization of the individual selected mycotoxins with different
228 combinations and concentrations of modifiers, showed the presence of $[M+H]^+$ as well as their
229 corresponding ammonium $[M+NH_4]^+$ and sodium $[M+Na]^+$ adducts. The addition of formic acid
230 (0.1 %) and 5 mM ammonium acetate increased the intensity of $[M+H]^+$ in the source instead of
231 $[M+Na]^+$ and $[M+NH_4]^+$ adducts. The study of SRM transitions confirmed that the sensitivity of
232 transitions with $[M+H]^+$ as a precursor ion was significantly higher in comparison with the peak
233 responses obtained for $[M+Na]^+$ and $[M+NH_4]^+$. Figure 1 illustrates the peak responses for the two
234 most sensitive SRM transitions using each precursor ion for beauvericin in the three studied
235 matrices, spiked at 1 µg/kg. In all cases, transitions from the $[M+H]^+$ showed higher sensitivity than
236 those coming from the adducts, specially the $[M+NH_4]^+$.

237 For both enniatins and beauvericin, at least three MS/MS transitions were proposed. For
238 beauvericin, as only two transitions from the protonated ion were feasible, a transition from the
239 sodium adduct was proposed for qualifying purposes. EnB1 and EnB showed the most sensitive
240 SRM transition from their corresponding protonated molecule to the product m/z ion 86.1. However
241 these transitions were not selected for quantification purposes due to the presence of higher noise
242 (Table 1).

243

244 3.2. Sample treatment.

245 Solid-liquid extraction with mixtures of acetonitrile/water (80:20, v/v) was initially tested,
246 following a similar protocol carried out in previous experiments with marine samples (Nácher-
247 Mestre et al., 2015). Extraction efficiency was studied comparing the signal obtained with spiked
248 samples and blank extract spiked prior injection at 1 µg/kg (62.5 ng/L in the extract for fish and
249 fillet, and 25 ng/L in the extract for feed). The addition of modifiers such as formic acid and/or
250 ammonium acetate in the extraction solvent was also tested. As illustrated in Figure 2, the use of

251 acidic extraction solvent decreased the signal obtained whilst ammonium acetate did not produce
252 significant changes in the extraction efficiency.

253 Non-polar enniatins and beauvericin presented lower recoveries (< 70 %) when water was
254 introduced in the extraction solvent, in comparison with the single use of acetonitrile. However, the
255 use of acetonitrile could promote the extraction of higher amounts of fatty acids, increasing matrix
256 effects. To minimize matrix effects (usually observed as signal suppression, Figure 3), the extracts
257 were diluted after sample extraction to minimize the interferences from the matrix and also, reduce
258 the percentage of organic content in the injected extract. Thus, chromatographic peak shape was
259 improved, avoiding the observed peak fronting in the most polar mycotoxins.

260

261 3.3. Method validation

262 Validation of analytical methods for fish feed, flesh fillets and whole fish samples was
263 carried out in accordance with the recommendations of the EU Scientific Opinion on the risks to
264 human and animal health related to the presence of beauvericin and enniatins in food and feed
265 (EFSA, 2014). Each matrix was validated at two concentration levels. Feed samples were validated
266 at 1 and 10 µg/kg, while flesh and whole fish were at 0.1 and 1 µg/kg. The validation of feed was
267 not feasible below 1µg/kg due to the absence of blank feeds (i.e. none of the feeds were mycotoxin
268 free). In addition, high interferences were found at low concentration levels in this complex matrix,
269 probably derived from the fatty content and pigments. Although the proposed method was not able
270 to selectively extract the mycotoxins, extracting also undesired matrix interferences, the dilution
271 step greatly helped to reduce signal suppression from the matrix.

272 Five replicates were prepared at each level. In addition, a sample blank and a procedure blank (only
273 solvents and/or reactants) were injected during the sequence of analysis.

274 Matrix-matched calibration showed good linearity in the range from 2.5 ng/L up to 10 ng/mL with
275 regression coefficients above 0.995 for both enniatins and beauvericin with residuals lower than 20
276 % and without any clear trend in their distribution. Table 2 shows the recoveries obtained for the

three studied matrices at the different spiked concentrations. Recoveries from the five replicates were above 70 % and below 109 % with maximum RSDs below 19 %. These results obtained are in agreement with recent articles on this discipline that also obtained satisfactory recoveries with acceptable deviations (Johny et al., 2019; Tolosa et al., 2019).

To deal with the specificity of the method, a blank procedure, a processed blank sample and a blank sample spiked at the lowest concentration (five replicates analyzed) were injected and compared. In this case, no peaks were found in the blanks selected for fish and whole fish samples and, therefore, the rules proposed in the validation section for specificity were satisfactory. This was not the case for feed where it was not feasible for EnA1, EnB, EnB1 and Bea, as no real blank was found from the large list of collected feed.

The limits of detection (LOD) were estimated by using the *S/N* ratios of 3, from the SRM chromatograms of samples spiked at the lowest concentration. LOD values ranged from 0.01 to 0.04 µg/kg while LOQ proposed were the lowest spiked concentrations with acceptable accuracy by applying the complete analytical method, 1 µg/kg for feed and 0.1 µg/kg for flesh and whole fillet (Table 2). Although LOD are not included in SANTE guidelines it gives an overview about the analytical capabilities of LC-MS/MS for detection at trace levels in complex matrices.

The wide majority of matrix-analyte combination suffered from matrix suppression (Figure 3). Higher matrix effects were observed for feed and whole fish, showing signal suppression higher than 50 % for beauvericin in feed and EnA in whole fish. Regarding fish fillet, matrix effects were lower, probably due to the lower presence of matrix co-extracted components such as lipids and proteins.

For accurate quantification, co-eluted matrix interferences must be minimized or compensated. Different approaches have been used to deal with matrix effects such as the dilution of samples and subsequently, minimizing the amount of co-eluted matrix introduced into the MS system (Decler et al., 2016; Panuwet et al., 2016; Renaud et al., 2017). However, dilution is limited as it reduces sensitivity and increases limits of detection. In the case of feed, 10-fold dilution was applied to

303 reduce co-eluted matrix and, 4-fold dilution was applied to fish samples. The use of isotopically
304 labelled analogue of the target analyte as internal standard is the most effective way to compensate
305 matrix effects (Panuwet et al., 2016). However, for the selected compounds, internal labelled
306 standards were not commercially available. Thus, quantification was carried out using matrix-
307 matched calibration, after applying the corresponding dilution.

308

309 *3.4. Application to sample analysis*

310 The proposed method was applied to the analysis of samples derived from feeding trials
311 carried out on sea bream and Atlantic salmon (further details of samples in Material and methods)
312 (De Santis et al., 2016; Hemre et al., 2016; Simó-Mirabet et al., 2018; Taylor et al., 2019; Vera et
313 al., 2019). Mycotoxins were detected in all the feeds. The highest values were found for Bea (80.4
314 µg/kg) while EnA was in all cases below LOQ. Bea, EnB and EnB1 were found in all feeds while
315 EnA1 was quantified in 20 feed samples with concentrations up to 3.8 µg/kg. EnB1 was found in
316 the concentration range between 2.4 - 10.9 µg/kg while EnB was between 8.0 - 32.8 µg/kg.

317 Neither whole fish nor fillet samples contained the studied mycotoxins at any detectable
318 concentration. Therefore, no feed to fillet transference of the parent compound was detected. This
319 evidence suggests no risk for human consumption but there could still be some concern that the
320 molecules studied might be metabolized and/or deposited in organs at concentrations below those
321 established in the current research (Anater et al., 2016; Glencross et al., 2019).

322 Figure 4 shows the SRM chromatograms from different sea bream and salmon feeds. Reliable
323 quantification and identification was carried out due to the acquisition of three SRM transitions with
324 q_i/Q ratios in agreement with values obtained from standards (SANTE, 2015).

325

326 **4. Conclusions**

327 Advanced analytical methodology based on the use of liquid chromatography coupled to
328 tandem mass spectrometry was developed to analyze the occurrence of emerging mycotoxins,

329 enniatins A, A1, B and B1 and beauvericin, in samples derived from feeding trials in farmed
330 Atlantic salmon and gilthead sea bream. The methodology proposed allowed the determination of
331 these emerging mycotoxins at low limits in complex fatty samples by acquiring three SRM
332 transitions per compound in a run time of only 6 min. The use of UHPLC-MS/MS resulted as an
333 extraordinary option to deal within medium-short list of target mycotoxins with short run time
334 analysis. Mycotoxins were found in feed at concentrations lower than 112 µg/kg (expressed as the
335 sum of the five mycotoxins). The results based on the use of new advanced analytical methodology
336 showed that samples of Atlantic salmon and sea bream reared on these feeds did not present any
337 detectable parent-mycotoxin, neither in the fillet (salmon and bream), nor in whole fish (salmon).
338 Therefore, no feed to fillet transfer of the parent mycotoxin compounds was detected.
339 Next challenges for the analysis of emerging mycotoxins will probably address the identification of
340 metabolites in target animal tissues where it is interesting to realize how can interact in biological
341 routes.

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349 **Conflict of interest statement**

350 The authors of this manuscript have declared no conflict of interest.

355 **References**

356

357 Anater, A., Manyes, L., Meca, G., Ferrer, E., Bittencourt Luciano, F., Pimpão, C. T., & Font, G.
358 (2016). Mycotoxins and their consequences in aquaculture: A review. *Aquaculture* 451, 1-10.

359 Beltrán, E., Ibáñez, M., Sancho, J. V., Hernández, F. (2009). Determination of mycotoxins in
360 different food commodities by ultra-high-pressure liquid chromatography coupled to triple
361 quadrupole mass spectrometry. *Rapid Communications in Mass Spectrometry*, 23, 1801-1809.

362 Beltrán, E., Ibáñez, M., Portolés, T., Ripollés, C., Sancho, J. V., Yusà, V., Marín, S., & Hernández,
363 F. (2013). Development of sensitive and rapid analytical methodology for food analysis of 18
364 mycotoxins included in a total diet study. *Analytica Chimica Acta*, 783, 39-48.

365 Decleer, M., Rajkovic, A., Sas, B., Madder, A., & De Saeger., S. (2016). Development and
366 validation of ultra-high-performance liquid chromatography–tandem mass spectrometry
367 methods for the simultaneous determination of beauvericin, enniatins (A, A1, B, B1) and
368 cereulide in maize, wheat, pasta and rice. *Journal of Chromatography A*, 1472, 35-43.

369 De Santis, C., Martin, S.A.M., Dehler, C.E., Iannetta, P.P.M., Leeming, D., Tocher, D.R. (2016).
370 Influence of dietary inclusion of a wet processed faba bean protein isolate on post-smolt
371 Atlantic salmon (*Salmo salar*). *Aquaculture*, 465, 124-133.

372 de Souza, M.L.M., Sulyok, M., Freitas-Silva, O., Costa, S.S., Brabet, C., Junior, M.M., Sekiyama,
373 B.L., Vargas, EA., Krska, R., & Schuhmacher, R. (2013). Cooccurrence of Mycotoxins in
374 Maize and Poultry Feeds from Brazil by Liquid Chromatography/Tandem Mass
375 Spectrometry. *The Scientific World Journal*, 2013, 427369.

376 EC, 2002. Directive 2002/32/EC of the European parliament and of the council of 7 May 2002 on
377 undesirable substances in animal feed. Off. J. Eur. Commun. L140, 10–22.

378 EFSA, 2011. Scientific Opinion on the risks for animal and public health related to the presence of

379 T-2 and HT-2 toxin in food and feed 1. EFSA Journal 9, 2481.

380 EFSA, 2014. Scientific Opinion on the risks to human and animal health related to the presence of
381 beauvericin and enniatins in food and feed EFSA Panel on Contaminants in the Food Chain
382 (CONTAM). EFSA Journal 2014; 12 (8):3802.

383 EU, 2006. Commission Recommendation No 2006/576 of 17 August 2006 on the presence of
384 deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products
385 intended for animal feeding. Off. J. Eur. Union L229, 7–9.

386 FAO, Food and Agriculture Organization of the United Nations, 2014. Economic Analysis of
387 Supply and Demand for Food up to 2030 – Special Focus on Fish and Fishery Products,
388 Rome, 2014. www.fao.org/publications/card/en/c/bd0f035b-f550-4246-bb8c-f0496faa0ccc

389 Glencross, B. D., Baily, J., Berntssen, M.H.G., Hardy, R., MacKenzie, S., & Tocher, D. R. (2019).
390 Risk assessment of the use of alternative animal and plant raw material resources in
391 aquaculture feeds. *Reviews in Aquaculture*. in press.

392 Hemre, G.I., Lock, E.J., Olsvik, P.A., Hamre, K., Espe, M., Torstensen, B.E., Silva, J., Hansen,
393 A.C., Waagbo, R., Johansen, J.S., Sanden, M., & Sissener, N.H. (2016). Atlantic salmon
394 (*Salmo salar*) require increased dietary levels of B-vitamins when fed diets with high
395 inclusion of plant based ingredients. *PeerJ*, 4, e2493.

396 Johny, A., Kruse Fæste, C., Borgevik, A.S., Berge, G.M., Fernandes, J.M.O., Ivanova, L. (2019).
397 Development and Validation of a Liquid Chromatography High-Resolution Mass
398 Spectrometry Method for the Simultaneous Determination of Mycotoxins and Phytoestrogens
399 in Plant-Based Fish Feed and Exposed Fish. *Toxins*, 11, 222.

400 Liland, N.S., Rosenlund, G., Berntssen, M.H.G., Brattelid, T., Madsen, L., & Torstensen, B.E.
401 (2013). Net production of Atlantic salmon (FIFO, Fish in Fish out < 1) with dietary plant
402 proteins and vegetable oils. *Aquaculture Nutrition*, 19, 289-300.

403 Malachová, A., Sulyok, M., Beltrán, E., Berthiller, F., & Krska, R. (2014). Optimization and
 404 validation of a quantitative liquid chromatography–tandem mass spectrometric method
 405 covering 295 bacterial and fungal metabolites including all regulated mycotoxins in four
 406 model food matrices. *Journal of Chromatography A*, 1362, 145-156.

407 Nácher-Mestre, J., Serrano, R., Beltrán, E., Pérez-Sánchez, J., Silva, J., Karalazos, V., Hernández,
 408 F., & Berntssen, M.H.G. (2015). Occurrence and potential transfer of mycotoxins in gilthead
 409 sea bream and Atlantic salmon by use of novel alternative feed ingredients. *Chemosphere*,
 410 128, 314-320.

411 Nácher-Mestre, J., Ballester-Lozano, G.F., Garlito, B., Portolés, T., Calduch-Giner, J.A., Serrano,
 412 R., Hernández, F., Berntssen, M.H.G., & Pérez-Sánchez, J. (2018). Comprehensive overview
 413 of feed-to-fillet transfer of new and traditional contaminants in Atlantic salmon and gilthead
 414 sea bream fed plant-based diets. *Aquaculture Nutrition*, 24, 1782-1795.

415 Panuwet, P., Hunter, R.E. Jr., D'Souza, P.E., Chen, X., Radford, S.A., Cohen, J.R., Marder, M.E.,
 416 Kartavenka, K. Ryan, P.B., & Barr, D.B. (2016). Biological Matrix Effects in Quantitative
 417 Tandem Mass Spectrometry-Based Analytical Methods: Advancing Biomonitoring. *Critical*
 418 *Reviews in Analytical Chemistry*, 46, 93-105.

419 Renaud, J. B., Kelman, M. J., McMullin, D. R., Yeung, K. K.-C., & Sumarah, M. W. (2017).
 420 Application of C8 liquid chromatography-tandem mass spectrometry for the analysis of
 421 enniatins and bassianolides. *Journal of Chromatography A*, 1508, 65-72.

422 SANTE, 2015. SANTE/11945/2015. Guidance document on analytical quality control and method
 423 validation procedures for pesticide residues and analysis in food and feed.

424 Shepherd, C. J., Monroig, O., & Tocher, D. R. (2017). Future availability of raw materials for
 425 salmon feeds and supply chain implications: The case of Scottish farmed salmon.
 426 *Aquaculture*, 467, 49-62.

427 Simó-Mirabet, P., Felip, A., Estensoro, I., Martos-Sitcha, J.A., de las Heras, V., Caldach-Giner,
 428 J.A., Puyalto, M., Karalazos, V., Sitjà-Bobadilla, A., & Pérez-Sánchez, J. (2018). Impact of
 429 low fish meal and fish oil diets on the performance, sex steroid profile and male-female sex
 430 reversal of gilthead sea bream (*Sparus aurata*) over a three-year production cycle.
 431 *Aquaculture*, 490, 64-74.

432 Sulyok, M., Krska, R., & Schuhmacher, R. (2007). A liquid chromatography/tandem mass
 433 spectrometric multi-mycotoxin method for the quantification of 87 analytes and its application
 434 to semi-quantitative screening of moldy food samples. *Analytical and Bioanalytical*
 435 *Chemistry*, 389, 1505-1523.

436 Taylor, J.F, Vera, L.M., De Santis, C., Lock, E.-J., Espe, M., Skjærven, K.H., Leeming, D., del
 437 Pozo, J., Mota-Velasco, J., Migaud, H., Hamre, K., & Tocher, D.R. (2019). The effect of
 438 micronutrient supplementation on growth and hepatic metabolism in diploid and triploid
 439 Atlantic salmon (*Salmo salar*) parr fed a low marine ingredient diet. *Comparative*
 440 *Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 227, 106-121.

441 Tolosa, J., Font, G., Mañes, J., & Ferrer, E. (2013). Natural occurrence of Fusarium mycotoxins in
 442 aquaculture fish food. *Revista de Toxicología*, 30, 193-197.

443 Tolosa, J., Font, G., Mañes, J., & Ferrer, E. (2014). Natural Occurrence of Emerging Fusarium
 444 Mycotoxins in Feed and Fish from Aquaculture. *Journal of Agricultural and Food Chemistry*,
 445 62, 12462-12470.

446 Tolosa, J., Rodríguez-Carrasco, Y., Ferrer, E., & Mañes, J. (2019). Identification and Quantification
 447 of Enniatins and Beauvericin in Animal Feeds and Their Ingredients by LC-QTRAP/MS/MS.
 448 *Metabolites*, 9, 33.

449 Vera, L.M., Lock, E.-J., Hamre, K., Migaud, H., Leeming, D., Tocher, D.R. & Taylor, J.F (2019).
 450 Enhanced micronutrient supplementation in low marine diets reduced vertebral malformation

in diploid and triploid Atlantic salmon (*Salmo salar*) parr, and increased vertebral expression of bone biomarker genes in diploids. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 237, 110327.

472 Table 1. Experimental MS/MS conditions for the studied mycotoxins.
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Mycotoxin	Retention time (min)	Precursor ion (m/z)	Product ion (m/z)	Collision energy (eV)	Cone voltage (V)	q ₁ /Q	q ₂ /Q
Enniatin B (EnB)	3.0	[M+H] ⁺ 640.2	196.0 (Q)	20	40	0.63	1.62
			214.3 (q ₁)	20			
			86.0 (q ₂)	60			
Enniatin B1 (EnB1)	3.3	[M+H] ⁺ 654.4	196.3 (Q)	60	40	0.57	1.41
			100.1 (q ₁)	30			
			86.1 (q ₂)	60			
Beauvericin (Bea)	3.4	[M+H] ⁺ 784.4	134.1 (Q)	50	40	0.40	0.39
			244.0 (q ₁)	30			
		[M+Na] ⁺ 806.5	384.2 (q ₂)	50			
Enniatin A1 (EnA1)	3.5	[M+H] ⁺ 668.2	100.1 (Q)	60	40	0.75	0.46
			210.0 (q ₁)	60			
			86.2 (q ₂)	20			
Enniatin A (EnA)	3.7	[M+H] ⁺ 682.4	100.0 (Q)	60	40	0.85	0.27
			210.0 (q ₁)	30			
			228.0 (q ₂)	30			

474 Q: quantification transition, q_i: qualitative transitions
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487 Table 2. Main recoveries (n=5), LOD and LOQ proposed for the studied mycotoxins in matrices from the aquaculture.

	Feed						Fish fillet						Whole fish					
	1 µg/kg		10 µg/kg		LOD (µg/kg)	LOQ (µg/kg)	0.1 µg/kg		1 µg/kg		LOD (µg/kg)	LOQ (µg/kg)	0.1 µg/kg		1 µg/kg		LOD (µg/kg)	LOQ (µg/kg)
	Rec (%)	RSD	Rec (%)	RSD			Rec (%)	RSD	Rec (%)	RSD			Rec (%)	RSD	Rec (%)	RSD		
EnA	70	8	70	19	0.04	1	100	13	85	5	0.01	0.1	98	8	107	14	0.01	0.1
EnA1	85	16	72	13	0.04	1	81	13	82	7	0.02	0.1	78	19	100	8	0.01	0.1
EnB	76	9	78	7	0.03	1	76	9	78	7	0.01	0.1	76	8	96	7	0.02	0.1
EnB1	95	11	77	3	0.03	1	87	11	91	8	0.02	0.1	99	19	93	14	0.03	0.1
Bea	93	19	72	14	0.03	1	86	11	92	10	0.03	0.1	104	7	109	11	0.03	0.1

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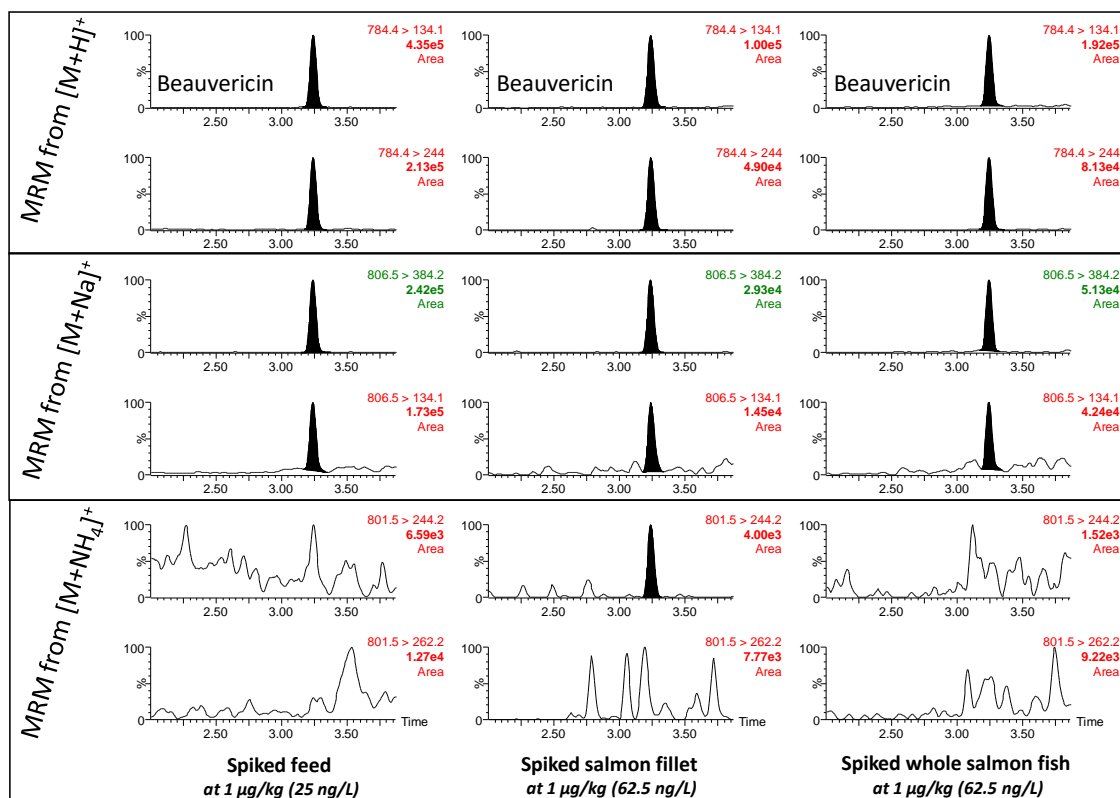


Figure 1. SRM chromatograms for beauvericin spiked at 1 µg/kg in feed, salmon fillet and whole salmon fish. Chromatograms for two selected SRM transitions are shown for each precursor ion ([M+H]⁺, [M+NH₄]⁺, and [M+Na]⁺) were illustrated for the three studied matrices.

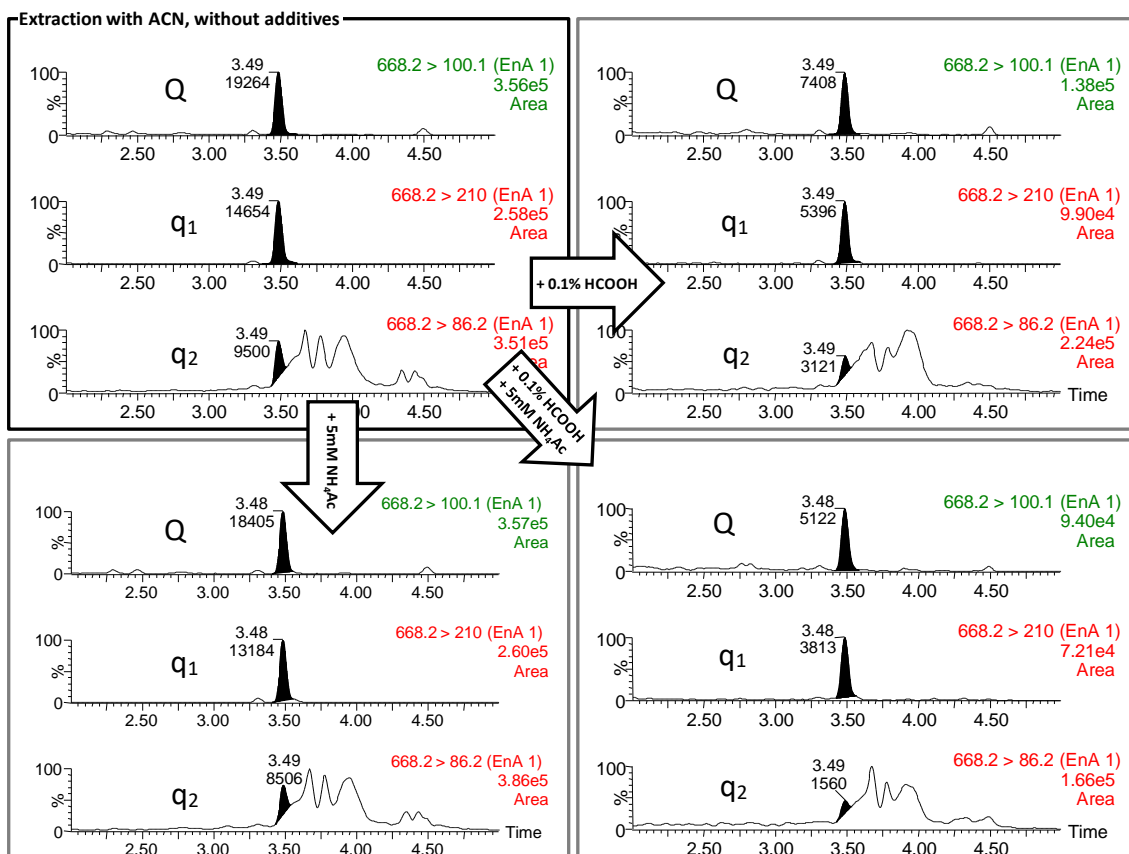
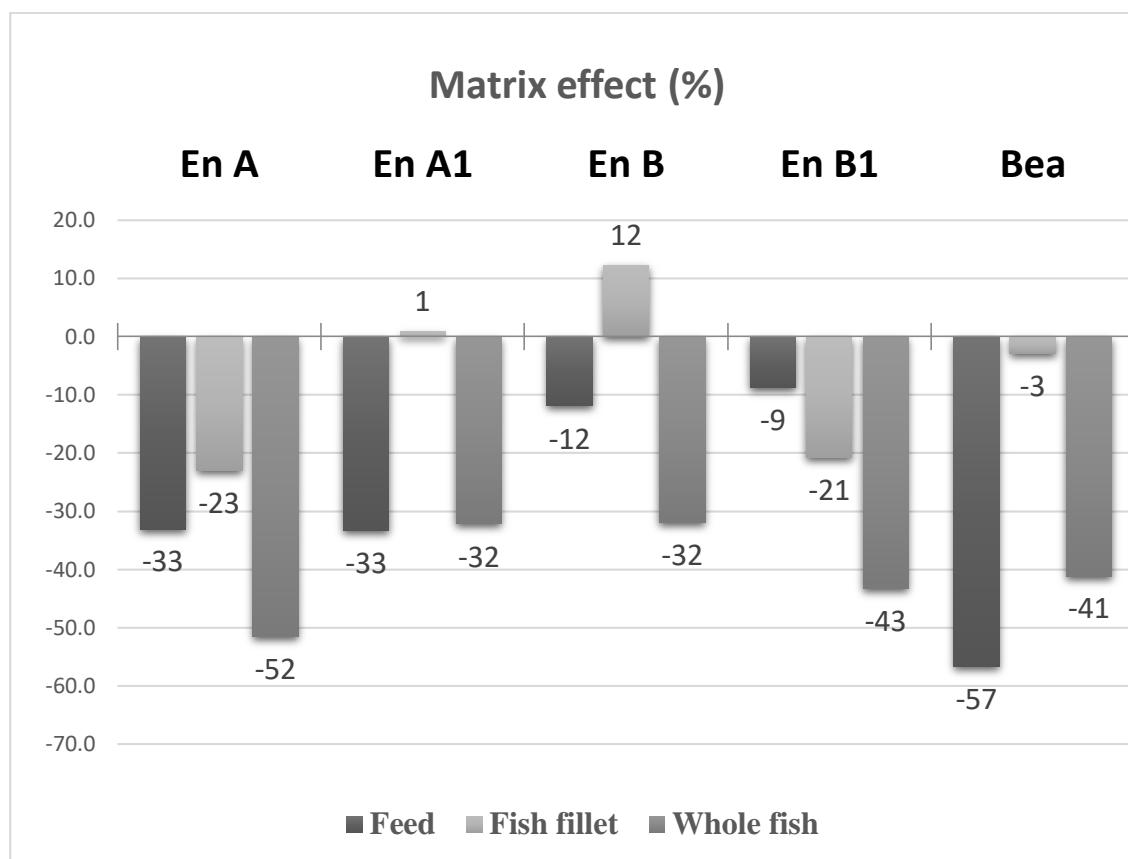


Figure 2. SRM chromatograms for EnA1 in feed spiked at 1 µg/kg. Effect on sensitivity produced by the addition of modifiers in the extraction solvent.



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517 Figure 3. Matrix effects calculated in percentage for mycotoxins in the three studied matrices.

518 Positive values indicated signal enhancement, whereas negative values were associated to signal
 519 suppression.

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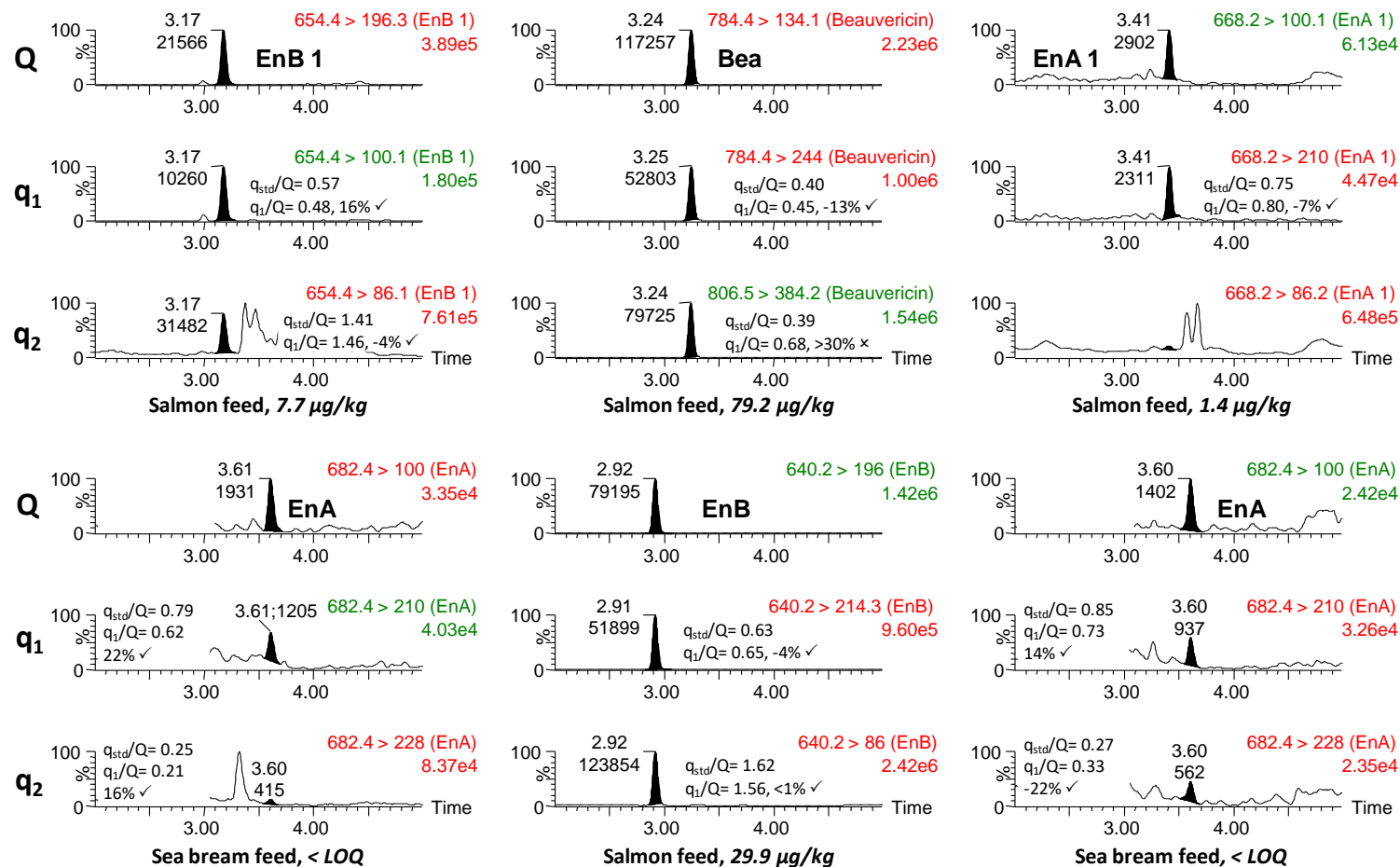
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529 Figure 4. SRM chromatograms for enniatins and beauvericin in feed samples. Experimental q_i/Q calculated in real samples were compared with the
 530 q_i/Q obtained injecting the standards in the same sequence.
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